Genetic Dissection of Behavior in Paramecium

Behavioral mutants allow a multidisciplinary approach to the molecular mechanisms of the excitable membrane.

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The machinery of even a simple behavioral pattern is difficult to analyze. We are approaching this problem by generating mutants that have altered behavioral responses. The organism we use is *Paramecium aurelia*, a unicellular animal that has sensory reception, an excitable membrane, and several motile responses. We have isolated over 300 mutant lines that are defective in the stimulus-response pathway. These mutations map at 20 genetic loci and cause several altered patterns of behavior. In addition to genetics and behavior, we have characterized the electrophysiological defects in these mutants by recording from an intracellular microelectrode.

Benzer (*1*) reasoned that the complex structures and events underlying behavior could be investigated by using behavioral mutants in which one element is altered at a time. Such genetic dissections of behavior have been carried out in genetically favorable species of flies, roundworms, and bacteria (*2*). The outstanding advantages of using *Paramecium* to study behavior is that both the genetics and the electrophysiology of this genus are well understood; these two fields of study have been brought together successfully in the project described herein.

Locomotor Behavior

Paramecia are completely covered by cilia, which beat actively toward the posterior end at a frequency of 10 to 20 hertz (*3*). This rapid beat propels the cell forward along a left-handed helical course. When disturbed, a paramecium responds with "avoiding reactions," first described by Jennings (*4*). In a typical reaction, the forward swimming is interrupted by a short period of backward swimming for a body length or more; then forward swim-

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ming is resumed, usually in a new direction. By a single avoiding reaction or a series of such reactions, that is, by trial and error, the cell escapes the chemical, thermal, or mechanical irritation. Backward swimming during the avoiding reaction is accomplished by reversing the direction of the power strokes of the ciliary beats.

The direction and frequency of the ciliary beat are controlled by electrical potentials across the cell membrane. There is a voltage difference (the resting potential) across the membrane such that the paramecium is electrically negative inside, as most other cells. Ciliary beat in the normal direction (toward the rear) is correlated with the resting potential and requires no excitation or impulse conduction (Fig. 1A). The level of the resting potential depends on the concentrations of the many cations in the bath (*5*, *6*), as predicted by the Goldman relation for a poorly selective membrane, according to Naitoh and Eckert (*5*,). Kinosita *et al.* (*7*) have shown that ciliary reversal, and hence backward swimming, are correlated with membrane depolarization. Such depolarization, or the appearance action potentials, is the result of the active properties of the membrane. In the action potentials of *Paramecium*, the inward current is carried by calcium ions (Ca^{2+}). Eckert (*8*) has proposed that this Ca^{2+} influx causes a transient increase in the intracellular Ca^{2+} concentration, which then causes the reversal of the ciliary beat (Fig. 1B). The action potential results from a voltage-dependent increase in Ca^{2+} conductance, that is, a Ca^{2+}-activation process, similar to the well-known sodium activation in nerves. The increase in Ca^{2+} conductance due to suprathreshold stimulations becomes regenerative; and either graded or all-or-none action potentials are seen, depending on the external ionic composition. The stimulations can be electrical, chemical, or mechanical in origin. In reaction to mechanical stimuli at the anterior end, receptor potentials can be generated, which are readily discernible from the action potentials they trigger. It is thus evident that the paramecium membranes have the same basic properties as excitable cells such as neurons and sensory cells. The molecular mechanism of the generation of action potentials and receptor potentials, and in particular the membrane proteins involved in ion gating, can be investigated by mutational analyses in this model system.

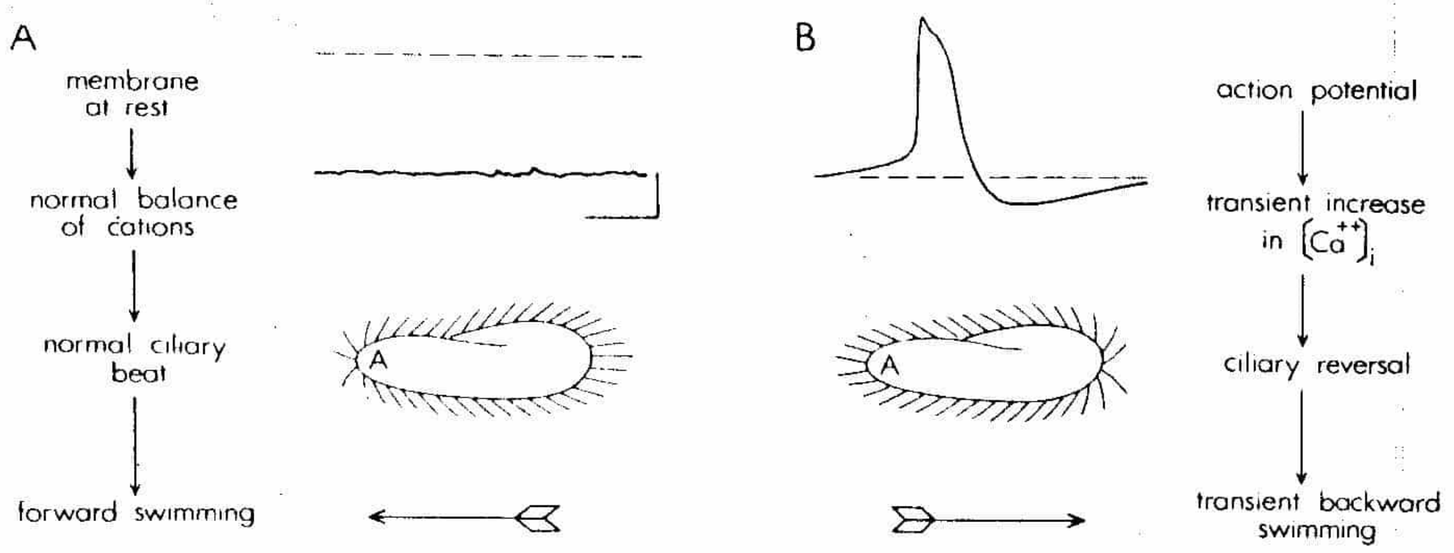

Fig. 1. Relation of membrane potential and locomotor behavior of *Paramecium*. (A) Top to bottom: Membrane potential at resting level. This level varies from −25 mv in the standard K-Ca solution to nearly zero in certain Ba solutions (4 m*M* KCl, 1 m*M* $CaCl_2$ with 1 m*M* tris, *p*H 7.2). A normal balance of cations is maintained, especially a very low internal Ca^{2+} concentration, $[Ca^{2+}]_i$. This state correlates with the normal ciliary beat in which the power stroke beats away from the anterior of the cell, *A*. The metachronized beat of thousands of cilia propels the paramecium forward (to the left). (B) Action potential in a stimulating $BaCl_2$ solution. The excited membrane allows entry of Ca^{2+} into the cell. The increased internal concentration of Ca^{2+} causes the cilia to beat in a reversed direction, hence the cell backs up. The situation quickly reverses when the active depolarization is over and excess internal Ca^{2+} is removed by active pumping. For details see Eckert (*8*).

Genetics and Isolation of Behavioral Mutants

The genetics of *Paramecium* has been well advanced under the leadership of T. M. Sonneborn (*9*). Paramecia can reproduce either asexually by cell division or sexually by conjugation between cells of two opposite mating types. Conjugation allows genic transfer and formal breeding analyses, as in most diploid eukaryotes. However, mutations can be induced, retrieved, and analyzed in *P. aurelia* much more easily than in other diploid species. This is because of a unique nuclear reorganization process called "autogamy," which represents an alternative form of sexual reproduction. Through autogamy, a cell proceeds from meiosis to a selective *haploid mitosis* to fertilization all with itself and all within a short span of a few hours. Since fertilization involves the fusion of two identical haploid nuclei, the cell becomes *completely homozygous* (*10*) (Fig. 2). Thus, one autogamy is equivalent to a long, long series of inbreeding or selfing in other diploid species. The end result is an organism that is truly diploid genotypically; but it expresses all of its genes phenotypically, including the recessive ones, since the two sets of genes are identical. Autogamy, like the rest of the life cycle, is well controlled in laboratories; and we have exploited it extensively in generating behavioral mutants, as described below.

We treated exponentially growing populations of *P. aurelia* (syngen 4) with the powerful mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and then induced nearly synchronous autogamy. In these mutagen-treated, exautogamous populations there was a small proportion, but a large variety, of mutants with abnormal growth rates, body shape, organelle structure, and behavior. Our major problem was deciding how to select from these populations the rare behavioral mutants we were interested in (the estimated mutation frequency was 10^{-4} for the dosage we used). Theoretically it would have been possible to select mutants by visually inspecting 10,000 or more cells (or the clones derived from them), but this technique would have been laborious and inefficient. Therefore, we devised various screening methods to find mutants with abnormal behavior of a certain type.

Our most successful methods to date exploit the conflicts between the animals' tendencies to swim upward (geotaxis) and to avoid ionic stimuli (chemotaxis). For example, we can construct a column containing a solution of relatively high Na^+ concentration, which generates repeated avoiding reactions in wild-type paramecia (*11*–*13*). When the mutagen-treated population is gently injected into the bottom of this column, the normal cells go into a fit of random backward and forward motion about the positions in which they are placed. The rare mutants that do not show avoiding reactions in Na^+ solution will swim up to the top of the column because of their geotactic response. Thus, the top fraction of the column contained the Na^+-insensitive mutants or generally insensitive mutants that we were seeking. A large number of mutants that were unable to achieve avoiding reactions were isolated this way. Variations of this scheme have been used to screen for other kinds of behavioral mutants (*14*).

While mutants or variants with subtle behavioral differences are plentiful, we have chosen to work only with those that have dramatic behavioral aberrations. With mutants that have clear-cut and dependable phenotypes, the physiological and genetic analyses are simpler and easier to interpret. The "misbehavior" we deal with can usually be identified by simple visual inspection even in the normal culture conditions. Most of the behavioral mutants selected in the past 5 years are listed in Table 1. The outstanding behavioral phenotypes are listed; these include abnormal speed and directions of movement,

and aberrant reactions to various stimuli. Some of the related physiological abnormalities will be described later. Although some mutants show pleiotropic effects such as subnormal growth rates or slight body deformation, these concomitant genic effects are apparently not the cause of the behavioral abnormalities.

Figure 3 shows the behavior of some of the mutants described in Table 1. These are the trajectories of paramecia moving in normal culture medium (*15*). Note that the wild type swims in helical paths but often stops and turns as a result of spontaneous avoiding reactions (Fig. 3A). "Fast-2" is a mutant that swims more rapidly than normal, especially when it is disturbed. Left undisturbed for a minute, each cell occasionally shows series of rapid avoiding reactions. The "pawn" mutant (named after the chess piece that can only move forward) lacks the avoiding reaction completely. The "paranoiac" mutant sometimes greatly exaggerates its avoiding reactions into long-distance backing (as if it thought it were being persecuted). The "sluggish" mutant stops or only barely creeps along slowly and thus registers little or no locomotion.

Systematic Alteration of Membrane Excitation

Electrophysiological investigation has revealed that most of the behavioral mutants have defects in their membrane functions. The electrophysiology of three mutants—pawns, paranoiac, and fast-2—will be described briefly and contrasted with that of the wild type to show how genic mutations can be used to modify systematically the electrical activities of the membrane.

As noted above, wild-type animals show frequent avoiding reactions in solutions with high concentrations of Na^+. This can easily be demonstrated by transferring the cells from a solution with no Na^+ (the adaptation solution of 4 m*M* KCl, 1 m*M* $CaCl_2$), to a Na^+ solution containing 4 m*M* NaCl and 1 m*M* $CaCl_2$ (*16*). Physiological experiments, in which the cell was secured and penetrated with a recording microelectrode, showed that each avoiding reaction is correlated with a depolarizing event. A typical depolarizing event is shown in Fig. 4. It lasts from 0.5 to 1 second and consists of a leading spike of about 20 millivolts followed by a series of oscillations near a slightly lower plateau (Fig. 4, center). Trains of these depolarization events appear 15 to 25 seconds after we change the bathing solution from the adaptation solution to the Na^+ solution. The nature of these active depolarizations is not completely understood. Although triggered by Na^+, the spike is apparently a calcium action potential because tetrodotoxin does not diminish the spike; and ciliary reversal, which is an indicator of Ca^{2+} influx, is activated.

Fast-2 is a mutant that does not avoid Na^+, although it avoids K^+ and Ba^+ when it first comes into contact with them. Physiologically, it does not give depolarization reactions when Na^+ solution appears in the bath. Instead, the level of resting potential drifts toward hyperpolarization (*17*). Figure 4 (left) shows the early lack of the normal response to the Na^+ solution.

Pawn, the mutant that completely lacks the avoiding reaction, shows depolarization episodes without spikes in reaction to Na^+ (Fig. 4, top). Although the spikeless episodes are fewer in number, the magnitude of the plateau potential, the duration, and the rate of fall of these episodes are

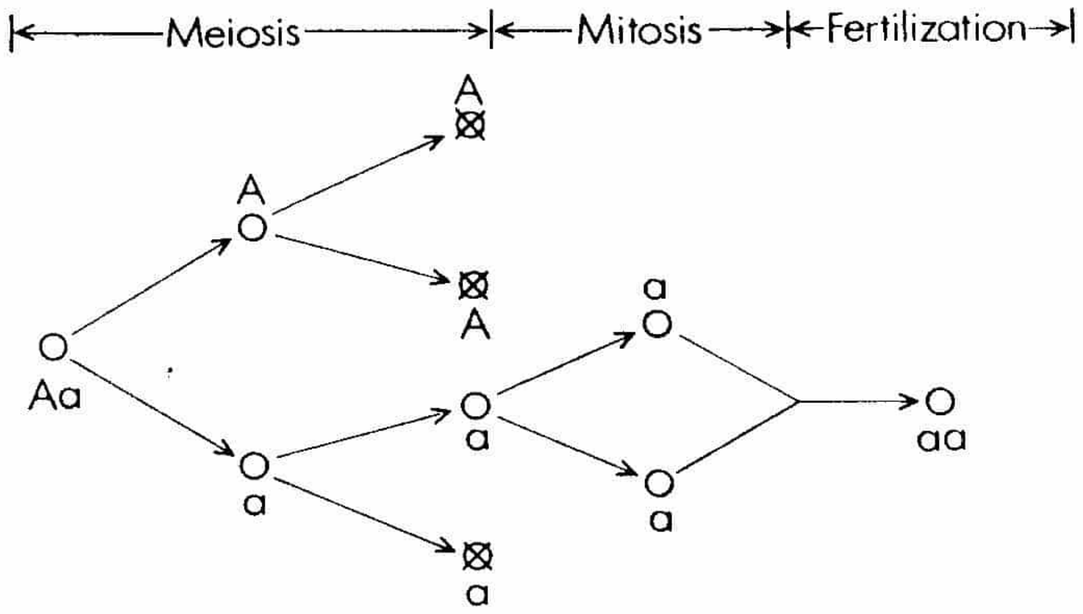

Fig. 2 (above). Reorganization of the germ nuclei in autogamy of *P. aurelia*. All but one of the meiotic products disintegrates. The remaining haploid nucleus replicates mitotically into two identical haploid copies. They then unite to restore diploidy. The consequence is a change of genotype to homozygosity in all the genes. This change takes place within the same cell without cell division. *Aa* marks the heterozygous state of one gene locus before autogamy. Here, homozygous recessive *aa* emerges after autogamy. Since the disintegration of the meiotic product is random, there is an equal probability that *AA* will emerge. For descriptions of the concomitant events of other nuclei and the techniques in preparing and inducing autogamies, see (*9*, *10*).

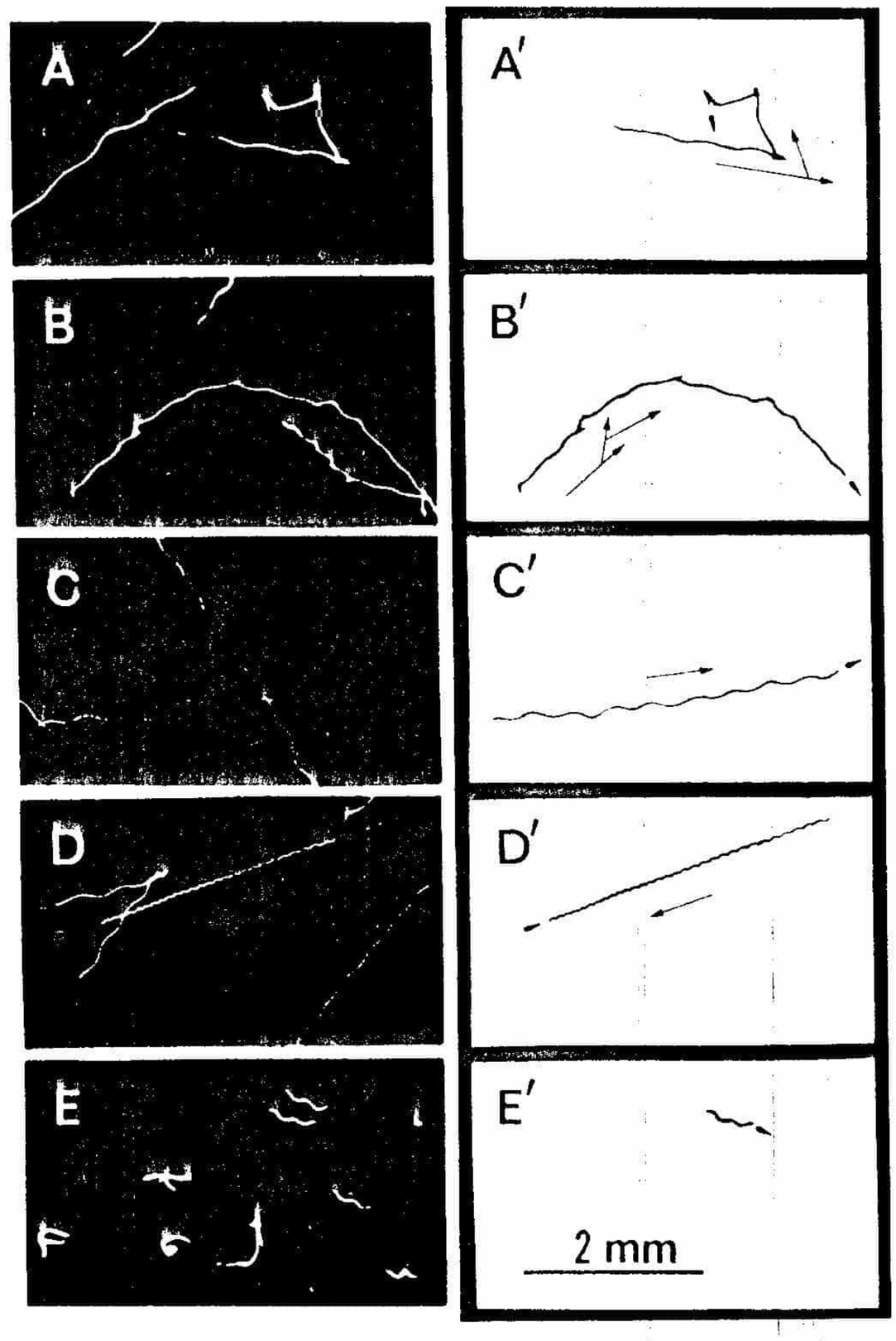

Fig. 3 (right). Tracks of various strains of paramecia swimming in normal culture medium. (A–E) Long-exposure, dark-field photomacrographs. Paramecia were left in culture medium for 1 to 3 minutes before the photographs were made. Each continuous line is the trajectory traversed by one paramecium during the 9.3 ± 0.1 second of film exposure. (A′–E′) Selected tracks of individual paramecia, demonstrating the behavior of each strain. A, A′; Wild type, showing helical path and spontaneous avoiding reactions which may cause a >90° turn. B, B′: Fast-2, with occasional series of rapid avoiding reactions causing <90° deviation of the forward path. C, C′: Pawn, with forward path never interrupted by avoiding reaction. D, D′: Paranoiac, with occasional sustained backward swimming characterized by rapid rotations and thus a tighter helix than the forward path. E, E′: Sluggish, with greatly retarded locomotion. See (*11*, *12*, *15*) for the methods used for photography and for determining of direction of movement.

similar to those of the spikes events in wild type. These spikeless depolarizations apparently are not related to the active influx of Ca^{2+}, because no ciliary reversal is ever observed in a typical pawn.

Paranoiac overreacts to Na^+ with prolonged depolarizations sustained at the plateau and lasting up to 60 seconds each (Fig. 4, right). As in the wild type, there is variation in the rate of depolarization. The downstroke, which repolarizes the membrane to the resting level after the sustained plateau, is much slower than that of the wild type. The sustained depolarization is closely correlated with the behavioral phenotype—the prolonged backward swimming when a paranoiac encounters Na^+.

While more complicated interpretations are available, the simplest hypothesis is to view the mutant patterns of electrogenesis as modifications of the wild-type pattern with single, point-mutational blockages (Fig. 4). Thus, the fast-2 mutation (*fna*) blocks the early depolarization event and thus abolishes the Na^+-response completely in the case of fast-2. The pawn mutation (*pw*) leads to a membrane defect that obstructs the generation of action potentials and thus modifies the wild-type pattern to the spikeless one of the pawn. The paranoiac mutation (*Pa*) blocks the normal repolarization process. Because of the lack of a proper shutoff mechanism, the membrane stays near the peak of depolarization for a prolonged period and eventually drifts slowly back to the resting level; hence, the prolonged backward swimming of paranoiacs (*18*).

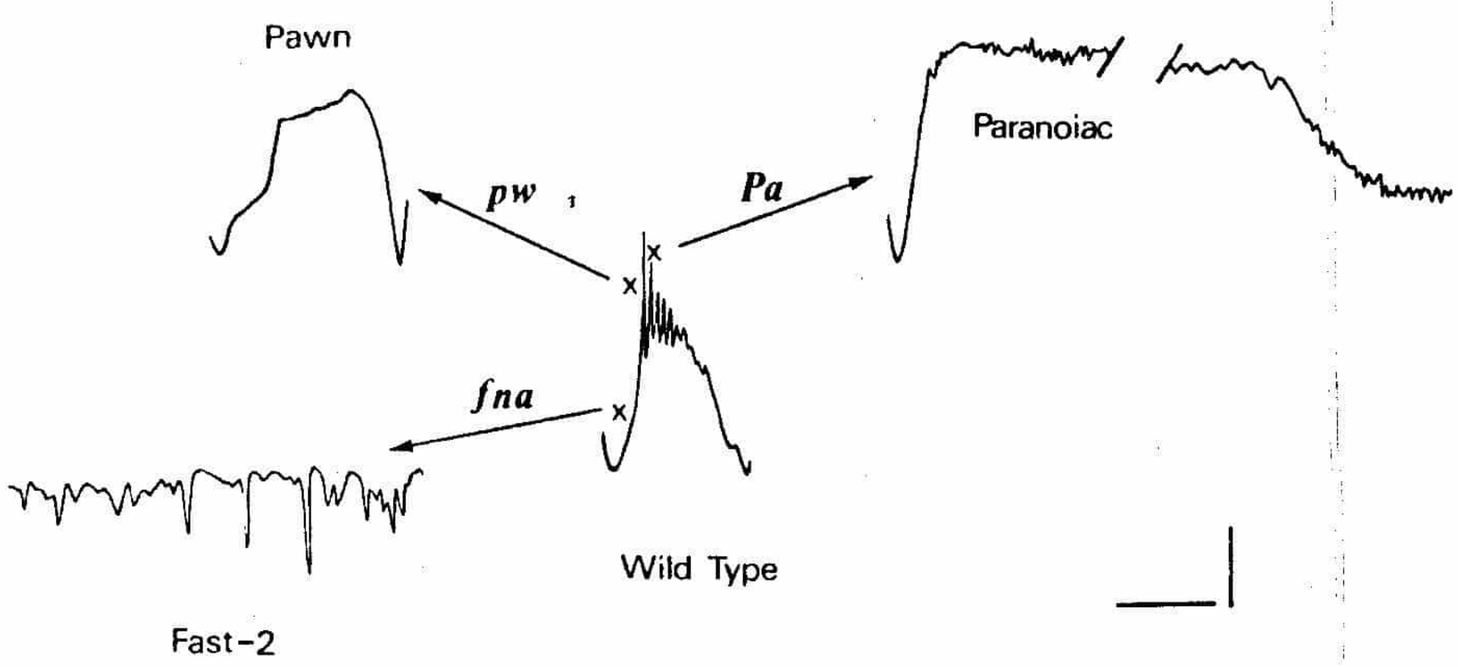

Fig. 4. Genetic modifications of active electrogenesis (*18*). Variant electrical activities can be viewed as impairments of the wild-type activity by single point blockages resulting from genic mutations. The center of the figure shows one episode typical of the recording from wild-type paramecium, in which there is a long series of electrical responses to the appearance of 4 m*M* NaCl, 1 m*M* $CaCl_2$, and 1 m*M* tris, *p*H 7.2, in the bath. In the case of the fast-2 mutant, blockage near the beginning of the depolarizing activity by the *fna* mutation prevents depolarization completely. Blockage at the upstroke of the action potential by the *pw* mutation leads to the spikeless pattern of the pawn mutant. Blocking the downstroke of the action potential by the *Pa* mutation sustains the depolarization near the peak level for a much longer duration. Broken lines mark the estimated resting levels. Calibration: 10 mv, 1 second.

Such a hypothesis has some predictions that have been tested. If one imposes any two or all three blockages onto the normal response, one would expect this response to be modified according to the temporal priority of the blockages. For example, a membrane with both *fna* and *Pa* defects should show only the *fna* effect and not the *Pa* one, since a defect in shutoff cannot be

Table 1. Phenotypes of the behavioral mutants in *P. aurelia* (syngen 4). Abbreviations: ts, temperature sensitive; TEA, tetraethylammonium chloride.

Class of mutant	No. of mutant lines collected	No. of independent mutations identified*	No. of gene loci identified	Behavioral phenotypes†
Pawn	146	58	2	Cannot swim backward; show no avoiding reaction to Na^+ or Ba^{2+} solutions or mechanical stimuli
ts-Pawn	5	4	2	Show wild-type avoidance response to ionic and mechanical stimuli when grown at 23°C; show no response above 35°C
Fast-1	10	4 + 2	2	Swim fast when disturbed in culture medium; show wild-type response to ionic and mechanical stimuli
Fast-2	19	3	1	Swim fast when disturbed in culture medium; swim fast and show no immediate avoiding reaction to Na^+ solution; show normal reaction to Ba^{2+} and TEA^+
Paranoiac	15	7	5	Move backward spontaneously for long periods of time in culture medium; show prolonged backward movement in Na^+ solution
ts-Paranoiac	2	0 + 2	?	Normal when grown at 23°C, paranoiac at 35°C
Spinner	54	11	1	Spontaneously spin upon hitting edge of dish; spin continuously in place in response to Na^+
Staccato	7	1 + 4	1	Show spontaneous, frequent avoiding reactions, dashing backward for some five body lengths during each avoiding reaction
Sluggish	5	1 + 3	1	Swim extremely slowly in all solutions if at all; give avoiding reactions in Na^+ and Ba^{2+} solutions
Atalanta	1	1	1	Stop transiently or show barely discernible backing in culture fluid or Ba^{2+} solutions
Chemotactic variant	4	1 + 3	1	Fail to accumulate in area of organic attractant; show wild-type responses to ionic stimuli
TEA^+-insensitive mutant	1	1	1	Show very few, weak avoiding reactions to Na^+ solution; show no avoiding reaction to TEA solution

*The first number in this column indicates the number of mutants of independent mutations. The independence is established through crosses and examinations of clonal origins. The second number, if present, shows the number of lines whose genetic relationship to others has not yet been analyzed. †The standard test solutions were as follows. Na^+ solution: 20 m*M* NaCl, 0.3 m*M* $CaCl_2$, 1 m*M* tris, *p*H 7.2; Ba^{2+} solution: 8 m*M* $BaCl_2$, 1 m*M* tris, *p*H 7.2; TEA^+ solution: 5 m*M* TEA, 1 m*M* $CaCl_2$, 1 m*M* tris, *p*H 7.2. Wild-type paramecia show repeated avoiding reactions in Na^+, Ba_2^+, and TEA^+ solutions.

shown unless the system is turned on in the first place. Such epistatic relationships of the three genes involved were studied behaviorally and physiologically in the three possible double mutants and the triple mutant. The results are totally consistent with the hypothesis (*19*).

We have examined some of the mutants in detail to identify their physiological defects (*20*). In fast-2, the relations between the resting membrane potential level and the external ionic concentration showed that it has an abnormally large K^+ permeability. Tetraethylammonium (TEA^+), a known K^+-channel blocker, cures most of the defects of fast-2. A different K^+ channel is mutated in the case of the TEA^+-insensitive mutant listed in Table 1. This mutant has a low input resistance and shows large K^+ leakage, which short-circuits the Ca^{2+}-activation mechanism in the generation of the action potential. Paranoiac was studied with current injected to induce the prolonged depolarization plateau, while recording from cells bathed in various Na^+ solutions. We also studied the ^{22}Na fluxes in paranoiac. The results suggest that paranoiac may have a defect in a Na^+ channel.

Much work has been done on pawns. These mutants that have lost their action potentials are very interesting and are described below.

Pawns: The Mutants with No Action Potentials

Although the well-known sodium hypothesis describes the ionic fluxes during an action potential, it is not at all clear what the membrane does to allow such fluxes. Specifically, no one has yet identified the molecular mechanism for the Na^+- or Ca^{2+}-activation that causes the influx of cations when the membrane is depolarized. To search for the membrane components that are involved, one may try to label them with specific pharmacological agents such as tetrodotoxin. We have labeled them instead with mutational alterations, which allow them to be identified by their differences from the wild-type components.

The pawns are mutants that have no calcium action potentials. The correlated behavioral phenotype, as described above, shows a loss of the ability to avoid stimuli that generate avoiding reactions in the wild type. Figure 5 is a dramatic demonstration of the pawn phenotype. Animals are added in a small drop to a solution containing a high concentration of barium ions (8 m*M* $BaCl_2$, 1 m*M* $CaCl_2$, represented as the dark field). Their movements during the first 13.3 seconds after this transfer are recorded in these long-exposure photomacrographs. Wild-type animals show repeated avoiding reactions in the Ba^{2+} solution and are thus confined to the vicinity of the area in which they are placed (see Fig. 5A). When pawns are subjected to the same test, no avoiding reaction is generated. The cells swim directly into the Ba^{2+} solution along their usual helical courses and give the sunburst pattern (Fig. 5B). The lower half of Fig. 5 shows the comparable intracellular records of the membrane potential of a wild type and a pawn specimen encountering the Ba^{2+} solution. In Ba^{2+} solution, unlike Na^+ solution, the wild type shows all-or-none action potentials (*6*). The train of action potentials corresponds to the series of avoiding reactions against Ba^{2+}. When the pawn encounters the Ba^{2+} solution, it shows a total lack of active electrogenesis. The lack of excitability in pawns can also be demonstrated by electric stimulations. Outward current injected through a stimulating electrode can evoke action potentials in the wild type, in a variety of solutions. Kung and Eckert (*21*) showed that injections of the same current failed to evoke action potentials in at least one of the pawns. The pawn membrane responds to the current electronically as a passive RC (resistance-capacitance) circuit. The passive properties of the membrane are apparently unaltered by the mutation. Input resistance, for example, as measured off the standard I-V (current-voltage) plot shows no difference between the wild-type paramecia and the pawn mutant. Our simple working hypothesis is that pawn mutations affect the voltage-dependent gating mechanism to Ca^{2+}.

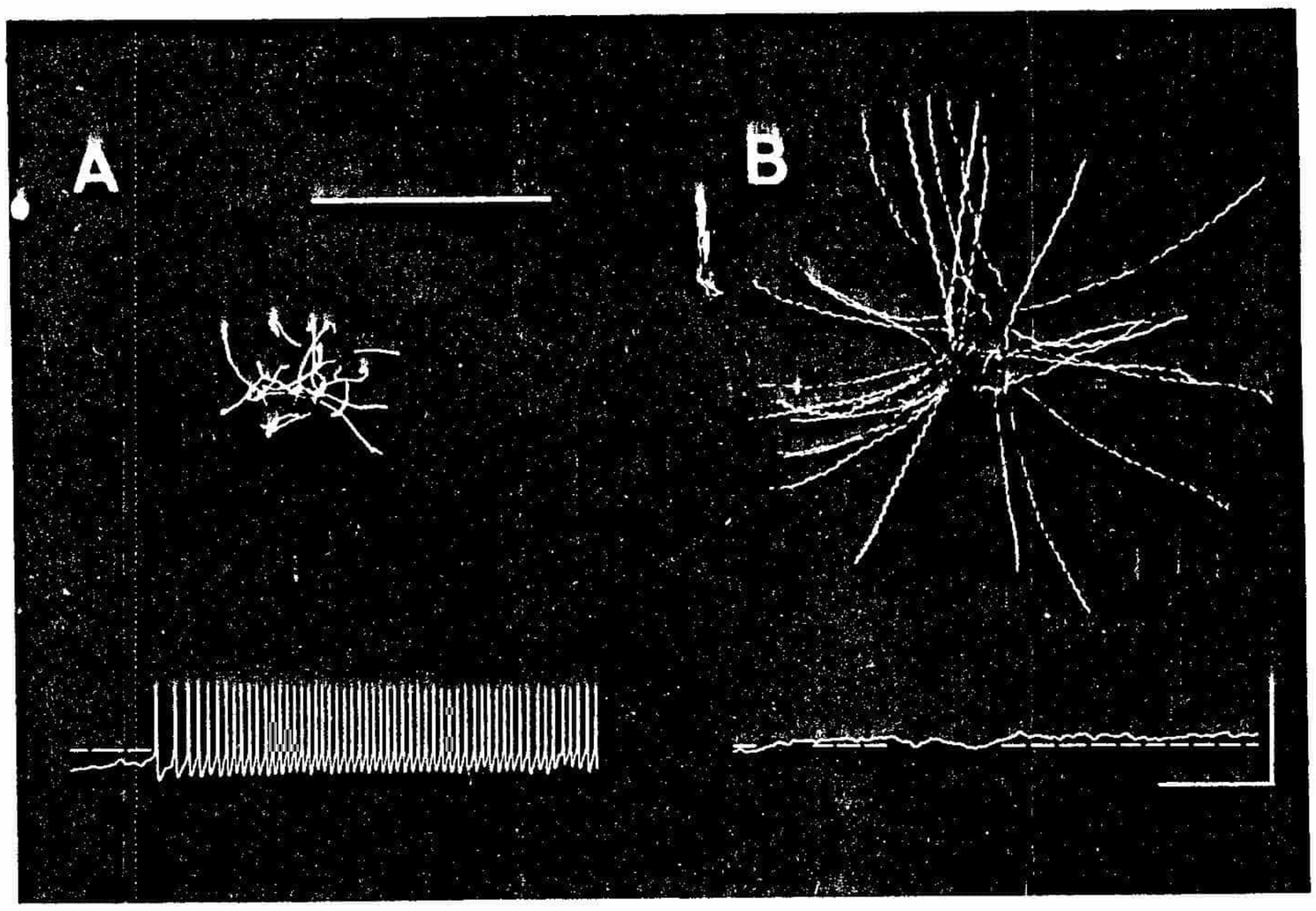

Fig. 5. Responses of wild-type paramecium (A) and pawn mutants (B) to Ba^{2+} solution. Top: Dark-field photomacrographs in which each continuous line records the movement of one paramecium during 13.3 ± 0.1 second immediately after transfer of animals into the Ba^{2+} solution. Because the wild-type cells repeatedly avoid the Ba^{2+} solution, they become confined to the vicinity of the area in which they were put. The pawn mutants swim into the surrounding Ba^{2+} solution with no avoiding reaction (scale, 1 cm). Bottom: Membrane potential of paramecia recorded in the Ba^{2+} solution. The membrane of the wild type (A) becomes depolarized to a near zero level (broken line) and trains of spontaneously generated all-or-none action potentials can be seen. While the pawn (B) membrane rests at approximately the same level, no action potential occurs. Experiments were performed at 23°C. The Ba^{2+} solution consisted of 8 m*M* $BaCl_2$, 1 m*M* $CaCl_2$, 1 m*M* tris, *p*H 7.2. Scale: 50 mv, 5 seconds.

Although we cannot be sure that the pawn gene product is indeed the structural protein of the Ca^{2+} gate, the gate is nevertheless defective in this mutant. If it is not the gate protein, the protein that is coded for by the normal genes on a pawn locus could be an element that directs the assembly of the gate or it might be an enzyme required for the synthesis of the lipids needed for normal gating of Ca^{2+}. We are searching for the proteins that correspond to the pawn mutations, and our effort has centered on resolving possible differences in membrane protein composition between the wild type and the pawn.

Regardless of how these proteins work, it can be demonstrated by the isolation of heat-sensitive pawn mutants that pawn genes encode macromolecules, presumably proteins, involved in the normal gating. Heat-sensitive mutations are those that affect the macromolecular gene products (ribonucleic acid or proteins) in such a way that they have a functional conformation at the low (permissive) temperature but not at the high (restrictive) temperature. Because of this, one sees the mutant

phenotype after the temperature is raised. We have succeeded in isolating mutants that are capable of excitation at the permissive temperature but lose their excitability at the restrictive temperature (*11, 22, 23*). Figure 6B shows the presence and absence of action potentials in one of these mutants grown at 23°C and 35°C, respectively. Excitation is not affected by the change of growth temperature in the wild type (Fig. 6A), and the excitability of the unconditional pawn cannot be restored by temperature shifts (Fig. 6C). The isolation of temperature-sensitive pawns not only shows the involvement of macromolecules in excitation and demonstrates that excitability can be made temperature-dependent, but it also provides a system of internal control in the study of excitability, since it can be turned on and off in identical cells in the same clone.

A total of 151 pawn mutant strains, including temperature-sensitive pawns, were found, which represent at least 62 independent mutational events. These mutations were mapped on three unlinked gene loci (*11, 22, 24*). Besides being important volumes in our "library" of membrane mutants, the pawns have a clear-cut and easily identifiable phenotype, making them very useful as genic markers in various crosses.

Can the behavioral defect (the inability to move backward) of pawns be due to another genic effect in the axonemal structure of the cilia? The answer is no. One can prepare models of paramecia treated with the detergent Triton X-100. These models have no functional membrane and presumably no permeability barrier. However, these models can be reactivated to swim forward in the presence of Mg^{2+}, K^+, and adenosine triphosphate. Even more amazing is the fact that these models can be reactivated to swim backward if sufficient Ca^{2+} is added to the solution. Models derived from pawns *can* swim backward as the wild type does (*25*). Thus, the inability of live pawns to reverse the beating direction of their cilia is not due to a mutation in the internal structure of the cilia, but is the result of the lack of Ca^{2+} entry; entry of Ca^{2+} is expected to occur after proper stimulation of the normal membrane.

Motility Mutants and Chemotactic Variants

Are there mutations that affect behavior not by membrane lesions but by their effects on the cilia? Mutants having flagella with ultrastructural defects are known in the flagellate *Chlamydomonas* (*26*). We have recently found a ciliary mutant of *Paramecium* whose structural defects are so subtle that no observable changes in their cilia have been seen under the electron microscope to date. This mutant, which we have called Atalanta, can swim forward normally but can only react to various stimulations with sudden stoppages (or barely discernible backing) along its forward paths. Unlike the case of pawn, this inability to sustain backing cannot be corrected by adding Ca^{2+} to swimming Triton-treated models of Atalanta. As far as we know, the reversal mechanism in the ciliary apparatus of Atalanta is irreversibly wrecked, although no ultrastructural damage has been detected. In contrast with pawn, the membrane of this mutant is not affected in all respects tested. Perfectly normal action potentials can be recorded from this mutant. Here, then, is a behavioral mutant whose behavioral deficiency is almost certainly due to the mutational damage of the cilia but not of the excitable membrane (*27*).

For a piece of behavior, such as the avoiding reaction, to have adaptive value, it must relate to the source of stimuli. *Paramecium* is known to respond to a variety of mechanical, thermal, and chemical stimuli. We have begun a project to reevaluate chemotaxis and chemoreception in *P. aurelia*.

To improve on the traditional methods of chemotaxis (*4, 28*), we have used a countercurrent flow tube (*29, 30*) and a T maze (*31*) to quantify chemotaxis. We found that wild-type paramecia are strongly attracted by acetate, lactate, and K^+, and weakly attracted by some other organic compounds (*31*). We also verified that they are attracted to weak acids (*p*H ~6) and are repelled by quinine (*28*). Paramecia presumably congregate in the area of attractant and leave the area of repellent through modulation of the frequency of the avoiding reaction (*4, 32*). Pawns provide a perfect test for this idea. The lack of avoiding reaction in pawn almost completely eliminates aggregation in potassium acetate. But, to our surprise, pawn retains most of the normal ability to stay away from quinine (*31*). We concluded that, at least in quinine repulsion and perhaps in all repulsion, other mechanisms besides avoiding reactions are involved (*32*).

Three chemotactic mutants were isolated. They show various degrees of loss of chemotactic response to attractants (*30, 31*). One of them, for example, is repelled by sodium acetate instead of being attracted by it as is the wild type. Such a specific behavioral aberration is probably due to loss of proper sensory reception of the attractants.

Evaluation of the Model System

We have shown that *Paramecium* provides a unique opportunity to study simple behavior. In these studies we have used and will continue to use techniques from four disciplines: (i) behavior; (ii) genetics; (iii) physiology; and (iv) biochemistry.

Behavior. The individual paramecium moves in a manner easily monitored with a low-power microscope. Its locomotion is

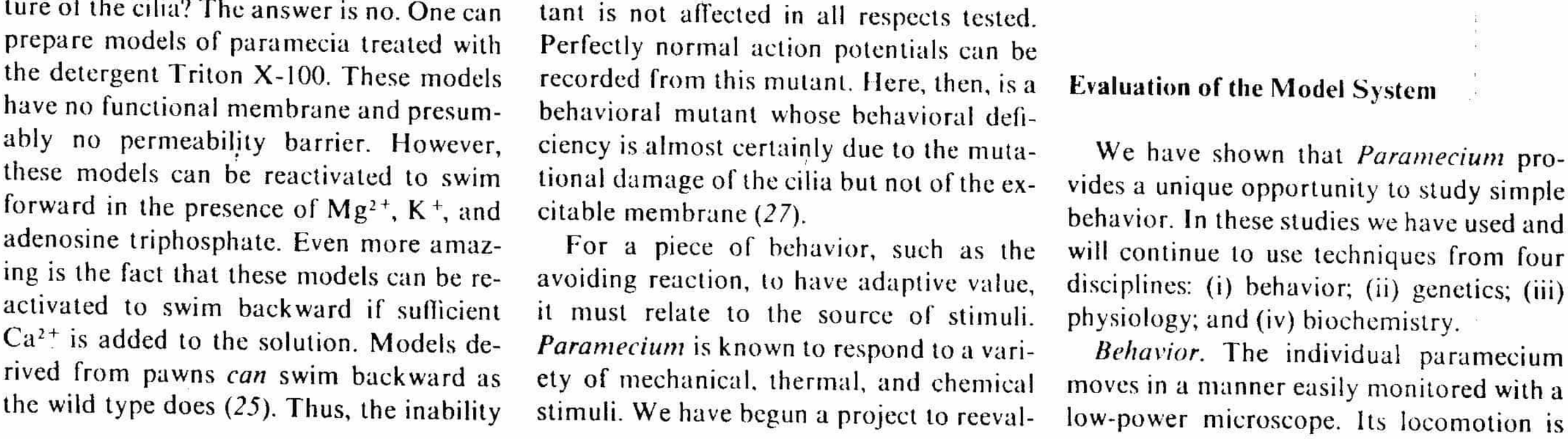

Fig. 6. Membrane potential records of a normal and two mutant strains of *P. aurelia*, after the Ba^{2+} solution entered the bath. (Left) Cells grown at 23°C. (Right) Cells grown at 35°C. A, A′: the trains of action potentials discharged by the wild type. B: The discharges of the temperature-sensitive pawn, d4-133, grown at 23°C. B′: The failure of excitation of d4-133 cultured at 35°C. C, C′: The lack of excitability of the temperature-independent pawn (d4-94). Broken lines mark the zero level. Inserts are records of higher speed, showing that the pattern of excitation is slightly aberrant in B. Calibration: 30 mv, 20 seconds (20 mv, 2.74 seconds for the inserts). [Courtesy of National Academy of Sciences (*23*)]

dictated by the form of ciliary beat, which reflects the state of the membrane. Therefore, we use behavior as an indicator of membrane function and malfunction. Populations of paramecia are manipulated to react to various stimuli, such as cations, gravity, and electric field in the laboratory. Failure to perform the proper "taxis" or "kinesis" distinguishes the mutants.

Genetics. Autogamy makes paramecium nearly as easy as haploid organisms to manipulate genetically. Conjugation, the alternative form of nuclear reorganization, involving a mating pair, permits genetic recombination and, thus, formal and rigorous genetic analyses. In addition to the mutants listed in Table 1, double and triple mutants have been constructed. These mutants make possible studies of the interaction of the multiple defects and the relations of the functional components.

Physiology. The very large size of *P. aurelia* (the cell measures up to 150 micrometers in length and 50 μm in width) permits the insertion of microelectrodes for conventional membrane physiological investigations. Passive properties, such as membrane potentials and resistances, and active properties, such as action potentials and receptor potentials, can be measured. These measurements in mutants and wild-type paramecia allow us to delineate various functional components of the membrane, for example, the Ca^{2+} gate or the K^+ channel. Recently, intracellular ionic content and fluxes of ions across the membrane have been studied by flame-photometry and the use of radioisotopes ^{45}Ca, ^{42}K, and ^{22}Na (*33*, *34*). Abnormalities in cation fluxes in mutants correspond to expectations based on other physiological observations.

Biochemistry. Since large masses of cells of the same genotype and phenotype can be obtained in a relatively short period, the chemistry of paramecium membrane is simpler and its results more clear-cut than that of other excitable membranes. The ciliary membrane is continuous with the plasma membrane and is very probably also excitable. Because cilia can easily be detached from the cell body, the ciliary membrane has been fractionated and purified. We have found that this surface membrane is unusual in its protein composition. Although there are many smaller proteins, the major membrane proteins have molecular weights greater than 200,000 (*33*). These proteins, known as the immobilization antigens, have been analyzed by other authors but their physiological functions have not been determined (*35*). We have found that they are not directly related to membrane excitation. With sodium dodecyl sulfate polyacrylamide electrophoresis we have not resolved any band differences related to some of the mutations discussed above.

Identification of the gene products important in the stimulus-response pathway is expected to have biological importance and will be a major part of our project. Although we have isolated mutants at the major steps of this pathway from sensory reception to motile response, the system is far from being saturated with mutations. There will be a continuing effort to use mutations to tag all the components in the complex machinery of this behavioral system. We will screen for mutants resistant or oversensitive to drugs or agents of known pharmacological and physiological function, similar to the TEA-insensitive mutants. We have shown in this article how the paramecium system can be exploited to study membrane excitation and behavior. The convergence of studies of behavior, electrophysiology, and genetics has been very fruitful. The search is now under way for the chemical identity of the various interesting gene products important in membrane functions related to behavior.

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10. In *P. aurelia* there are two germ nuclei (micronuclei) per cell. One of them is omitted in Fig. 2 because all four of its miotic products disintegrate and do not contribute to the germ line. There is an 860-ploid somatic nucleus (the macronucleus) in each cell for the vegetative function of this large unicell. It disintegrates into fragments while the germ nuclei replicate and is eventually lost. The zygotic nucleus (the new germ nucleus, *aa* in Fig. 2) divides and the genomes replicate to fulfill the set of one macro- and two micronuclei per cell descended through postautogamous cell division. For details see (*9*).
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13. The solution in the column most suitable for the isolation of mutants insensitive to cations consists of: 20 m*M* NaCl, 0.3 m*M* $CaCl_2$, 1 m*M* tris (hydroxymethyl) amino methane, *p*H 7.2. To prevent mixing when the cells are injected at the bottom of the column, 2.2 percent sucrose is added to the culture medium of the mutagen-treated population before injection.
14. The column can be filled with other solutions designed to screen for different mutants. For example, when a solution rich in tetraethylammonium chloride ions (TEA^+, known to block the K^+ permeability in paramecium) was used, we isolated a strain of mutants which failed quite specifically to avoid TEA^+. The column can also be used "upside-down." When it was filled with a solution which encourages geotaxis and the mutagenized population was layered on top, the fraction collected at the bottom after a few minutes was enriched with "paranoiacs."
15. The dark-field photomacrography used is similar to that of S. Dryl [*Bull. Acad. Pol. Sci. Ser. Sci. Biol.* **6**, 429 (1958)]. A Polaroid version of this method was successfully adopted as described by Chang and Kung (*12*). We dimmed the light at the first second of film exposure and thus the fainter trace at one end of the track marks the starting segment. Such fainter traces are often lost during photographic reproduction for publication.
16. All salt solutions also contain 1 m*M* tris(hydroxymethyl)aminomethane (tris) buffer at *p*H 7.2. The adaptation solution also contains 1 m*M* citrate known to preserve the vigor of the specimen.
17. The fast-2 phenotype is complicated. The resting potential drifts to a hyperpolarized level after Na^+ solution appears in the bath. After 5 to 10 minutes the resting level of depolarization is restored and then trains of all-or-none spikes can often be recorded. These trains of sharp spikes correlate with the series of rapid avoiding reactions shown in Fig. 3B. The membrane of this mutant also showed spike-like hyperpolarizations (Fig. 4) much more frequently than wild-type membrane. The initial hyperpolarizing drift is also observed in Ca^{2+} solution; this drift appears to be the response to solutions lacking K^+.
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36. This project began with the encouragement and supervision of Dr. T. M. Sonneborn. Dr. R. Eckert supervised the electrophysiological study of the first mutant. Supported by NSF grant GB-32164X and PHS grant GM-19406 to C.K.